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Junction Potentials, Electrode Standard Potentials, and Other Problems in Interpreting Electrical Properties of Membranes

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Summary. As background to a detailed analysis of the cation permeation mechanism in rabbit gallbladder epithelium, this paper considers several general problems in interpretation. With regard to liquid junction potentials, the common practice of using saturated KCl bridges was insufficiently accurate for the present purposes because the resulting junctions are time-dependent and poorly understood theoretically. Time-independent and well-defined junction potentials were obtained by arranging all junctions to be of the biionic or single-salt dilution types. The magnitudes of these junction potentials were estimated in three different ways, with good agreement. Recording arrangements using either agar bridges or else Ag/AgCl electrodes also yielded good agreement after appropriate corrections for junction potentials and electrode potentials. The effects of nonelectrolytes on electrode standard potentials were measured. Two experiments were devised to determine whether transepithelially measured electrical properties of the gallbladder refer to a single membrane or to two membranes in series: the potential difference change resulting from a mucosal concentration change was measured as a function of the serosal concentration, and intracellular concentrations were altered by increasing bathing solution osmolalities with an impermeant nonelectrolyte. Both types of experiment indicated that transepithelial measurements are dominated by a single membrane. Small corrections were applied to measured potential differences to take account of unstirred-layer effects with permeant salts.

The purpose of this and two following papers is to characterize the mechanism of alkali cation permeation in a biological membrane.

The ideal system for such an analysis would be a single cell membrane which is easy to dissect and tolerant of a wide range of solution conditions, which maintains constant properties for long times *in vitro*, and which offers large selectivity differences among the alkali cations, low anion permeability, a single cation permeation pathway, properties independent of solution composition and time, absence of "electrogenic" ion pumps, and ready access to the solutions on both sides with minimal unstirred layers. No

biological system fits this description perfectly, but, as will be seen, gallbladder epithelium is satisfactory and advantageous in several respects. The alternative available systems provided by perfused single excitable cells, such as squid axon, have the disadvantage of permeability properties which undergo transient changes and perhaps reflect multiple cation permeation pathways. Analysis of the far more complex problems posed by these excitable systems may become easier when permeation in nonexcitable systems such as the gallbladder is better understood.

This first paper is devoted in large part to approaches for dealing with three general problems that complicate attempts to obtain accurate electrical measurements of ion permeability in any membrane: the simple question of design of experimental solutions, and the thornier and usually neglected problems posed by junction potentials and by the effects of nonelectrolytes on standard potentials and activity coefficients. The remainder of this paper presents evidence that transepithelial measurements of electrical properties in the gallbladder are dominated by a single membrane, even though the structure of the whole epithelium is, of course, more complex. The following papers (Wright, Barry & Diamond, *in preparation*; Barry, Diamond & Wright, *in preparation*) will report the experiments on the mechanism of cation permeation.

Methods

The techniques used for obtaining *in vitro* preparations of rabbit gallbladder and for measuring transepithelial electrical potential differences (p.d.'s) were similar to those described previously by Diamond (1962*b*, 1964), Diamond and Harrison (1966), and Wright and Diamond (1968). Briefly, the gallbladder was removed from anesthetized male white rabbits (5 to 6 lb), everted, cannulated with a polyethylene cannula, filled with a salt solution, and transferred to a 30-ml beaker of solution at room temperature. In the everted orientation, the single cell layer that constitutes the epithelium is in direct contact with the external bathing solution (referred to as the mucosal solution), whereas the layer of connective tissue and muscle fibers about 300-μ thick faces the luminal bathing solution (referred to as the serosal solution). In all experiments, the external solution was stirred with a stream of oxygen bubbles, and the serosal solution was left unstirred. The structure of the gallbladder is illustrated in Fig. 1, and the question what route permeating ions follow is discussed on pp. 109–116.

The p.d.'s were recorded on a Keithley 610B electrometer connected to a Varian G11A potentiometric chart recorder. As will be discussed in more detail on pp. 97–107 the electrodes were either Ag/AgCl electrodes dipping directly into the bathing solutions or (more often) calomel half-cells ($Hg/HgCl_2$, saturated KCl) connected to the bathing solutions by polyethylene bridges filled with 4% agar and appropriate salt solutions. Since the salt transport mechanism of the gallbladder is an electrically neutral pump that produces no p.d. (Diamond, 1962*b*, 1968), and since active transport was in any case minimal or zero under the experimental conditions used [(23 °C, no bicarbonate bathing solutions either Na^+- or K^+-free) (Diamond, 1968)], the p.d.'s resulting from

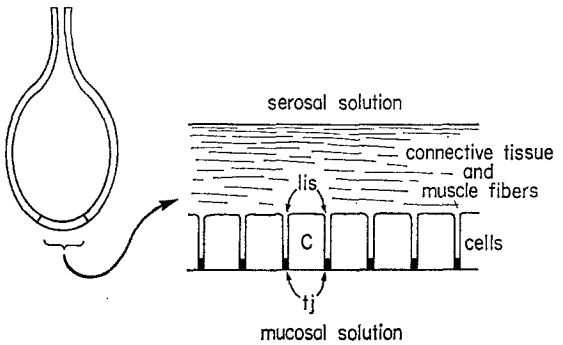

Fig. 1. Schematic diagram of gallbladder structure (not to scale). The everted gallbladder (left) is a sac in which a single uninterrupted layer of epithelial cells (*C*, right) on the outside is supported by a layer of connective tissue and muscle fibers on the inside. At the face abutting on the mucosal solution, the cell membranes of adjacent epithelial cells are fused together in so-called tight junctions (*tj*), beyond which the cells are separated by lateral intercellular spaces (*lis*), which are open at the end facing the connective tissue and serosal solution. Although in cross-section the tight junctions appear as spots, they actually constitute hoops completely circling the base of each barrel-shaped epithelial cell. Thus, ions crossing the gallbladder must either permeate through the tight junction and then diffuse down the lateral intercellular spaces (i.e., crossing a single membrane) or enter the cells across the cell membrane facing the mucosal solution and then leave the cells across the cell membrane facing the lateral spaces and connective tissue (i.e., crossing two membranes in series)

isoosmotic changes in ion concentration gradients are to be interpreted wholly as diffusion potentials unaffected by active transport.

Stable and reproducible Ag/AgCl electrodes were prepared from silver wires coated with an epoxy resin except for an exposed tip of 4 to 5 mm. The silver was cleaned in concentrated HNO_3 and then washed in running distilled water for one to three days. The electrodes were then chlorided with currents of about 1 to 2 mA/cm^2 for 30 min in 0.1 M HCl, without reversing the direction of the current, and were finally washed for 6 to 8 hr in running distilled water before testing. Electrodes were made up in batches of six, and only pairs of electrodes differing in potential by less than 0.2 mV in 150 mM salt solutions were used.

A Beckman cationic glass electrode no. 39137 (NAS 27-4) was used to study effects of nonelectrolytes on standard potentials (p. 107). This electrode is sensitive in varying degrees to H^+ and to all the alkali cations. Since the measurements were made in pure KCl solutions, only H^+ could interfere with the response to K^+, and the H^+ concentration was therefore reduced to a low value by saturating the solutions tested with $Ca(OH)_2$. The electrode was presoaked in 0.1 M KCl before use.

Gallbladders were generally dissected in a solution composed of 148 mM NaCl, 6 mM KCl, 0.25 mM $CaCl_2$, 2.125 mM Na_2HPO_4, and 0.345 mM NaH_2PO_4. Next, to check that the preparation was in good condition, several measurements were obtained of the diffusion potential resulting from a 2:1 NaCl concentration gradient (referred to as a "dilution potential" – in contrast to "biionic potentials" which result from cation concentration differences at constant anion concentration, such as 150 mM NaCl vs. 150 mM KCl). Gallbladders in which the initial value of the 2:1 NaCl dilution potential was less than 8 mV were rejected. In all diffusion potential measurements, it was the composition of the mucosal solution that was transiently changed, while the composition

of the serosal solution was held constant. After this measurement in NaCl, both bathing solutions were changed to a solution of the particular salt being studied in the given experiment. For a change of salt on both sides of the epithelium, the mucosal bath (in a 30-ml beaker) was changed at least twice while maintaining constant stirring. Because of the diffusion delays associated with the connective tissue on the serosal side, the everted sac was initially washed out five or six times with fresh solution and then three or four more times after a wait of about 5 to 15 min. When both bathing solutions were changed symmetrically in this fashion, a diffusion potential transiently appeared across the gallbladder because of the time required for the solution at the serosal face of the epithelium to change from the composition of the old bathing solution to that of the new bathing solution, due to the diffusion delay in the connective tissue. When this transient p.d. had decayed back to about 1 mV or less (after 15 min on the average), indicating equilibration of the serosal solution up to the epithelium, the experiment was continued. All p.d.'s are given as the potential of the serosal solution with respect to that of the mucosal solution.

All experiments were conducted at an ambient room temperature of 23 ± 1 °C.

Experimental Solutions

The quantitative interpretation of electrical measurements becomes increasingly more difficult, the more ion species are present. In principle, it would have been ideal to have been able to do all the experiments in solutions containing only a single alkali halide salt or salt pair. In practice, the simplest solution ensuring good survival of the gallbladder must contain, in addition to a single alkali halide, small amounts of Ca^{++} and a H^+ buffer. Ca^{++} is necessary to maintain the epithelial cell layer structurally intact. Since Ca^{++} has a high affinity for membrane sites and competes with alkali cations for them so that the electrical properties of the gallbladder show marked Ca^{++} dependence (Diamond & Harrison, 1966; Wright & Diamond, 1968; Wright *et al. in preparation*), $[Ca^{++}]$ was kept arbitrarily constant at a value chosen to maximize cation-anion discrimination by including 0.25 mM $CaCl_2$ in all solutions. Similarly, the electrical properties of the gallbladder show pH dependence because of the affinity of H^+ for membrane sites (Wright & Diamond, 1968), so all solutions were buffered at pH 7.4 ± 0.1.

In a few experiments, the buffer used was 3 mM Tris (hydroxymethyl aminomethane + HCl). For most experiments, however, the buffer chosen was 2 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, molecular weight 238.3), which had the advantage of much higher buffering capacity because the desired experimental pH (7.4) was much closer to the pK_a of HEPES (7.55 at 20 °C) than to the pK_a of Tris (8.1). The HEPES acid at 2 mM was neutralized to pH 7.4 with approximately 1 mM of the appropriate alkali hydroxide (e.g., RbOH for RbCl solutions, CsOH for CsCl solutions, etc.). Comparisons in the same gallbladder showed that there were no differences between the electrical properties of the gallbladder whether solutions were buffered with Tris or HEPES.

Except in a few types of experiments cited specifically, all solutions were designed to have an osmolality of 283 ± 4 mosm, as checked with the Fiske osmometer. The concentration of alkali halide was generally 150 mM except in solutions used to measure dilution potentials, which generally contained 75 mM alkali halide. This partial removal of an alkali halide to measure a dilution potential was balanced by addition of an isosmotic amount of mannitol, calculated by using the osmotic coefficient of 1.00 for mannitol at the concentrations used and osmotic coefficients for alkali halides from Robinson and Stokes (1965). All solutions were made up molal (i.e., concentration

are in millimoles solute per kg water), since the fractional solution volumes occupied by mannitol and the alkali halides were not negligible and use of molar solutions (millimoles solute per liter solution) would have led to inconsistencies.

Impurities in commercially available alkali halide salts can be a serious problem in selectivity studies, and in the course of looking for suitable grades of chemicals we encountered, in particular, low-grade RbCl samples containing enough acid contaminants to exceed the buffer capacities. We finally settled upon the following sources and grades: CsCl (99.9% pure) and RbCl (99.8% pure) from Penn Rare Metals Division of Kawecki Chemical Co., KCl (99.8% pure) and NaCl (99.9% pure) from Baker Chemical Co., and LiCl (>99.9% pure) from Fisher Scientific Co.

Junction Potentials

To determine the p.d. across a membrane separating two bathing solutions of different ionic composition, one may record either with identical electrodes dipping directly into the two different bathing solutions or with identical electrodes contacting identical solutions and connected to the experimental bathing solutions indirectly via identical salt bridges. To obtain the transmembrane p.d. from the p.d. of the whole circuit, one must subtract the difference in electrode potentials in the former case and the difference in liquid junction potentials in the latter case. Thus, any attempt to measure and interpret transmembrane p.d.'s must first come to grips with the complicated problems of junction potentials and electrode potentials. These problems were particularly acute in the present study of rabbit gallbladder, across which the membrane potentials resulting from ion concentration gradients were generally less than 12 mV (always less than 30 mV), whereas the junction potentials encountered were up to 9 mV. Hence this section of text will discuss three questions: (1) why the common practice of "eliminating" junction potentials by means of saturated KCl bridges is unsatisfactory for accurate work; (2) what arrangements can be adopted to yield stable junction potentials or electrode potentials; and (3) how to calculate values of these junction potentials or electrode potentials so that they may be subtracted from the circuit p.d. to yield the transmembrane p.d. Further discussion will be found in chapter 13 of the book by MacInnes (1961; *see* also Teorell, 1953, & Caldwell, 1968).

Saturated KCl Bridges

It is common practice to use saturated KCl bridges to reduce junction potentials to small values. For the following two reasons, this procedure introduces uncertainties of up to 2 to 5 mV in the case of free-flowing bridges and up to 10 mV for static bridges, and therefore becomes unsatisfactory if higher accuracy is desired or if the transmembrane p.d.'s are small.

(1) Even if concentration profiles in the junctional region quickly reach and remain in a steady state because the two solutions are kept perfectly stirred up to near the interface – a necessary condition for obtaining a stable junction potential calculable from the Planck equation – the value of this junction potential is not reduced to zero by use of saturated KCl. Furthermore, although these KCl junction potentials offer practical advantages due to their small size, their actual values are subject to some uncertainty, since the worst agreement between theoretical and experimental junction potentials arises for junctions involving KCl (MacInnes, 1961, p. 237). These difficulties are illustrated in Fig. 2, which compares junction potentials between various LiCl-CsCl mixtures as determined in four ways by saturated KCl bridges without applying any correction for the KC

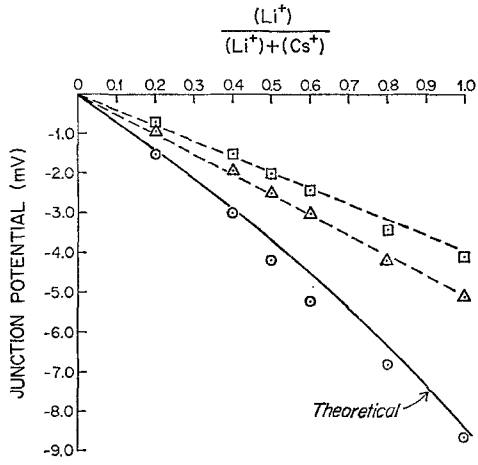

Fig. 2. Junction potentials for LiCl-CsCl mixtures estimated in four different ways The junction considered is 150 mM CsCl vs. a LiCl-CsCl mixture with a $[Cl^-]$ of 150 mM and the $(Li^+)/[(Li^+)+(Cs^+)]$ ratio given on the abscissa. The ordinate gives the junction potential (potential of CsCl solution with respect to mixture solution). The four methods were as follows. (1) The unbroken line gives the theoretical p.d.'s calculated from a modified Planck-Henderson equation (Eq. (2)). (2) The points (⊙) were measured with Ag/AgCl electrodes and corrected according to the Guggenheim assumption, as discussed in Table 2 and on p. 104. (3) The points (□) were measured with saturated KCl agar bridges by the circuit calomel:saturated KCl:saturated KCl in agar:150 mM LiCl-CsCl 150 mM CsCl in agar:150 mM CsCl:saturated KCl in agar:saturated KCl:calomel subtracting the slight p.d. with the solutions 150 mM CsCl:150 mM CsCl to correct for any electrode asymmetry. These points would give correct values if the potentials of the saturated KCl junctions were actually zero, which they are not. (4) The △ points differ from the □ points in that the theoretical values for the two saturated KCl junctions (saturated KCl:150 mM LiCl-CsCl and 150 mM CsCl:saturated KCl) have been calculated from a modified Henderson equation (Eq. (2)) and subtracted from the circuit p.d. Note that this still leaves a considerable discrepancy compared to the first two methods, owing to inadequate theoretical understanding of KCl junctions resulting in particularly large errors with saturated solutions

junctions, by saturated KCl bridges after subtracting the theoretical values of the KCl junctions, by calculation from a modified Planck-Henderson equation (Eq. (2) & Appendix), and by measurement with Ag/AgCl electrodes after activity corrections according to the so-called Guggenheim assumption (*see* p. 104). Although the latter two methods agree closely (*see* also p. 106), the uncorrected values obtained from KCl bridges are in error by up to 4.5 mV, and errors of up to 3.5 mV persist even after correction for the somewhat unsatisfactory theoretical values of the KCl junctions.

(2) If the two solutions are not kept stirred up to near the interface, a more serious difficulty appears, namely, the value of the junction potential changes with time. This difficulty is certain to occur with static salt bridges in which the solution is immobilized by agar, and may also appear with microelectrodes unless the tip is sufficiently coarse and the applied pressure sufficiently high to ensure an adequate flow rate of saturated KCl solution out the tip of the electrode (but such a flow may have the disadvantage of damaging the preparation or changing the composition of the intracellular fluid or bathing solution). There are only two situations in which the value of a junction potential is independent of time in a static junction: for two solutions of the same salt at different concentrations (e.g., 150 mM NaCl vs. 75 mM NaCl, a so-called dilution junction), and for solutions

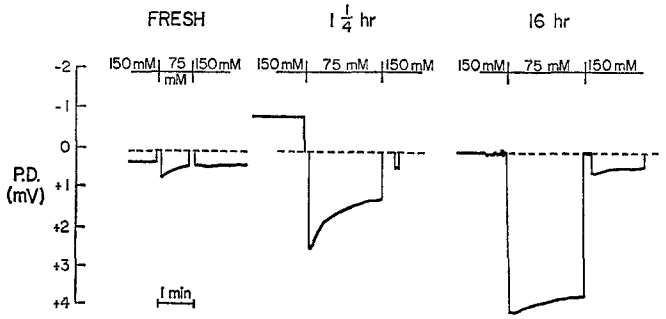

Fig. 3. The asymmetry p.d., $\Delta E_{II} - \Delta E_{I}$, between two saturated KCl salt bridges transferred from 150 mM LiCl to 75 mM LiCl and back to 150 mM LiCl, as a function of their different histories. The opposite end of each bridge was connected to a reservoir of saturated KCl solution, the p.d. being measured via calomel half-cells. In each case, bridge I had been taken freshly out of saturated KCl, and bridge II either had been taken freshly out of saturated KCl (first trace), or had been stored for $1\frac{1}{4}$ hr (second trace) or 16 hr (third trace) with one end in a 150 mM LiCl solution (the opposite end was kept in saturated KCl). The broken line represents zero p.d., the value expected for fresh saturated KCl bridges or for bridges with identical histories. Note that the value of the asymmetry p.d. varies between 0 and 4 mV as a function of the bridge's history, reflecting a junction potential between 75 mM LiCl and the 150 mM LiCl – saturated KCl mixture actually in the tip of bridge II. This figure illustrates the potential danger in using saturated KCl bridges for exact measurements of dilution potentials

of two different salts at the same concentration and having either the anion or the cation in common (e.g., 150 mM NaCl vs. 150 mM KCl, or 150 mM NaCl vs. 150 mM NaF, a so-called biionic junction) – provided in both cases that the ionic mobility ratios are independent of concentration. In a static junction composed of two different salts at different concentrations (e.g., saturated KCl vs. 150 mM NaCl), the value of the junction potential changes with time as the form of the profile changes. In addition, and even more serious, during measurements in a particular bathing solution, the ionic composition in the tip of a static bridge will tend to approach that of the bathing solution, and the junction potential in a new solution will now no longer be that expected for saturated KCl but will depend upon the history of the bridge. For instance, Fig. 3 shows that when a saturated KCl bridge is kept for 1 hr (or 16 hr) in 150 mM LiCl and is then transferred to 75 mM LiCl, the junction potential shifts by 3 to 4 mV – i.e., one no longer has the small value expected for saturated KCl:75 mM LiCl, but something approaching the small value for saturated KCl:150 mM LiCl in series with the large value for 150 mM LiCl:75 mM LiCl. Fig. 4 illustrates a similar

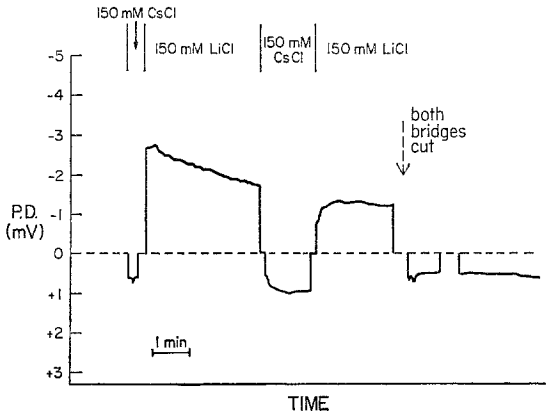

Fig. 4. The asymmetry p.d., $\Delta E_{II} - \Delta E_{I}$, between two saturated KCl salt bridges transferred from 150 mM CsCl to 150 mM LiCl, as a function of their different histories. The opposite end of each bridge was connected to a reservoir of saturated KCl solution, the p.d. being measured via calomel half-cells. Bridge I had been taken freshly out of saturated KCl, and bridge II had been stored with one end in 150 mM CsCl for $2\frac{1}{2}$ hr (the opposite end was kept in saturated KCl). The broken line represents zero p.d., the value expected for fresh saturated KCl bridges or for bridges with identical histories. The asymmetry p.d. was measured successively in 150 mM CsCl, 150 mM LiCl, 150 mM CsCl, and 150 mM LiCl. The p.d. in LiCl was initially 3.3 mV and decayed with time: this represents the junction potential between 150 mM LiCl and the 150 mM CsCl – saturated KCl mixture actually in the tip of bridge II. When the tips of both bridges were cut back by 5 cm to expose fresh saturated KCl in agar, the asymmetry p.d. was reduced nearly to zero. This figure illustrates the potential dangers in using saturated KCl bridges for exact measurements of biionic potentials

shift for a saturated KCl bridge transferred to 150 mM LiCl after storage for $2\frac{1}{2}$ hr in 150 mM CsCl.

For these reasons, saturated KCl bridges were not used in the present study.

Arrangements to Yield Stable Junction Potentials

For alkali halide dilution potentials [$M^+X^-(C_1)$ vs. $M^+X^-(C_2)$, where C_1 was generally set at 150 mM and C_2 at 75 mM], each bathing solution was connected to a calomel half-cell via a polyethylene bridge filled with the given salt at 150 mM in 4% agar. For instance, LiCl bridges were used for LiCl dilution potentials, CsCl bridges for CsCl dilution potentials, etc. The chemical part of the circuit may be written as calomel electrode: saturated KCl:M^+X^- (150 mM) in agar bridge:M^+X^- (150 mM):gallbladder:M^+X^- (75 mM):M^+X^- (150 mM) in agar bridge:saturated KCl: calomel electrode. Thus, only the value for the junction M^+X^- (150 mM): M^+X^- (75 mM) need be subtracted from the circuit p.d. to obtain the transmembrane p.d.

For alkali halide biionic potentials [$M^+X^-(C_1)$ vs. $N^+X^-(C_1)$, where C_1 was generally 150 mM], each bathing solution was connected to a calomel half-cell via an agar bridge of either M^+X^- or N^+X^- at 150 mM. For instance, either KCl or NaCl bridges were used for the KCl:NaCl biionic potential. Only the value for the junction M^+X^- (150 mM):N^+X^- (150 mM) need be subtracted from the circuit p.d. to obtain the transmembrane p.d.

Both the dilution junction and the biionic junction offer the advantage that the value of the p.d. is nearly independent of the junction concentration profile and hence of time (completely independent if mobility ratios are independent of concentration). An additional advantage of this recording arrangement is that the bridge in contact with the serosal bathing solution always remains constant in composition, whereas the mucosal bridge is exposed to solutions of differing composition only briefly. For instance, the procedure to measure the NaCl:KCl biionic potential across the gallbladder was to record with 150 mM NaCl bridges, to maintain the serosal solution as 150 mM NaCl, and to take the mucosal solution through the sequence 150 mM NaCl → 150 mM KCl → 150 mM NaCl. Thus, only the mucosal bridge is exposed to a differing solution, that exposure is brief (usually $\leqq 1$ min), and the bridges retain their original composition up to near the tip.

Calculation or Measurement of Junction Potentials and Electrode Potentials

In this section we discuss how to obtain the potentials of the dilution junctions and biionic junctions described in the previous paragraph.

It is impossible to measure directly the potential of a single junction or the activity coefficient (γ) of a single ion; i.e., some assumption about single-ion γ's must be made to extract a junction potential from any experimental measurement, or vice versa. It would be erroneous to conclude from this fact, however, as some workers formerly did, that junction potentials and single-ion γ's are without physical meaning or that their values are very uncertain. One can cross-check different combinations of values of junction potentials and γ's obtained from quite different methods, such as experimental methods based on the recently developed cation-sensitive glass electrodes (Garrels, 1967), measurements based on Ag/AgCl electrodes, and calculation from the Planck or Henderson equations. We shall compare here three methods or sets of assumptions for estimating the desired junction potentials: calculation from a modified form of the Planck and Henderson equations, measurement by Ag/AgCl electrodes interpreted according to the so-called MacInnes assumption, and measurement by Ag/AgCl electrodes interpreted according to the so-called Guggenheim assumption. All three methods yield potentials agreeing within 1 mV for all but one of the junctions studied (Table 2).

Method 1. Junction potentials were calculated from the Planck equation modified to take activity coefficients into account. For dilution junctions and biionic junctions, the Planck equation (*see* MacInnes, 1961, p. 233) and the Henderson equation (MacInnes, 1961, p. 231) reduce to the same form.

In the Appendix, it is shown that the "dilution" junction potential E_L between two solutions of the same univalent-univalent salt at different activities a' and a'' is given by the expression

$$E_L = -\frac{RT}{F}\frac{(u_1-u_3)}{(u_1+u_3)}\ln\frac{a''}{a'} \tag{1}$$

where u's are mobilities, subscript 1 refers to the cation, and subscript 3 to the anion. The derivation uses the Guggenheim assumption (p. 104) that the anion and the cation have the same activity coefficient ($\gamma_+ = \gamma_- = \gamma_\pm$), but Eq. (1) holds regardless of whether γ is independent of concentration and is thus constant through the junction.

The "biionic" junction potential [$M^+X^-(C_1)$ vs. $N^+X^-(C_1)$] is shown in the Appendix to be given by the equation

$$E_L = -\frac{RT}{F}\left[\frac{a_2''(u_2-u_3)-a_1'(u_1-u_3)}{a_2''(u_2+u_3)-a_1'(u_1+u_3)}\right]\ln\frac{a_2''(u_2+u_3)}{a_1'(u_1+u_3)} \tag{2}$$

Table 1. *Comparison of theoretical junction potentials at 23 °C, based on activities and on concentrations*[a]

Concentration gradient (mM)		Theoretical junction potential (mV)		
Side 1	Side 2	Using activities	Using concentrations	Error
50	100	3.76	4.05	0.3
50	200	7.49	8.11	0.6
50	400	11.19	12.16	1.0

[a] The junction considered is that between two NaCl solutions at the different concentrations given in the first two columns. The theoretical values of the junction potentials listed in column 3 were calculated from Eq. (1) in the text, which uses activities. The values listed in column 4 were calculated from the unmodified Planck-Henderson equation, which approximates activities with concentrations. (The potentials in columns 3 and 4 are those of the concentrated solution with respect to the dilute solution.) The last column gives the difference between the third and fourth columns, i.e., the error introduced by using concentrations.

where subscripts 1 and 2 refer to the two cations, 3 to the anion. The derivation assumes that $\gamma_1 = \gamma_2 = \gamma_3$ at any point, but γ need not be constant through the junction.

Eqs. (1) and (2) have the same form as the Henderson or Planck equations but contain activities rather than concentrations. As seen in Table 1, which compares theoretical values of NaCl dilution junctions calculated from Eq. (1) and from the Henderson-Planck equation, the use of concentrations instead of activities introduces an error of up to 1.0 mV.

Since Eqs. (1) and (2) were to be evaluated using ion mobilities in free solution, it was necessary to establish if mobility ratios are the same in an agar bridge as in free solution. This was done by measuring the p.d. between an agar bridge and a coarse-tipped (ca. 50-μ) free-solution microelectrode, both containing saturated KCl and dipping into KCl solutions of various concentrations. The microelectrode was found to become 0.26 mV more negative than the bridge per 10-fold decrease in solution concentration. The difference in the K^+ transport number between an agar bridge and free solution implied by this p.d. is only 0.002 (~0.4%) and may be neglected.

Since ion mobility ratios vary with concentration for some salts, we equated the mobility ratios u_K/u_{Cl}, u_{Na}/u_{Cl}, and u_{Li}/u_{Cl} with the transport-number ratios at 100 mM determined by Longsworth (1932) [in a solution of a single univalent-univalent salt, the transport numbers are related to the mobilities by the definitions $t_+ = u_+ C_+/(u_+ C_+ + u_- C_-)$ and $t_- = u_- C_-/(u_+ C_+ + u_- C_-)$]. A concentration of 100 mM was chosen as intermediate between 75 and 150 mM, the usual concentrations in our junctions. For LiCl

and NaCl, the mobility ratio at infinite dilution (the ratio of the limiting equivalent conductances) differs sufficiently from the ratio at 100 mM so that use of the former would have introduced an error of up to 0.6 mV into calculated junction potentials. Since the cation transport number for KCl changes by less than 0.1 % between 0 and 100 mM, and since u_{Rb} and u_{Cs} are even closer to u_{Cl} than is u_K, the values of u_{Rb}/u_{Cl} and u_{Cs}/u_{Cl} at infinite dilution were used.

Substitution of these mobility ratios into Eqs. (1) and (2) yielded the values in the last column ("theoretical p.d.") of Table 2 for the junctions used in the present study.

Methods 2 and 3. Junction potentials were also determined experimentally by means of Ag/AgCl reference electrodes. To estimate a dilution junction potential, we measured the p.d. of the circuit $Ag/AgCl:M^+Cl^-$ (150 mM):M^+Cl^- (150 mM) in agar bridge:M^+Cl^- (75 mM):Ag/AgCl. Biionic junction potentials were estimated from the p.d. of the circuit $Ag/AgCl:M^+Cl^-$ (150 mM):M^+Cl^- (150 mM) in agar bridge:N^+Cl^- (150 mM):Ag/AgCl. In each case the junction potential is given by the measured p.d. minus the calculated difference in the electrode potentials $E=E_0-(RT/F)\ln a_{Cl}$ at the two Ag/AgCl electrodes, where E_0 is the standard state potential and $a_{Cl}=\gamma_{Cl}\,C_{Cl}$ involves the single-ion activity coefficient γ_{Cl}.

Mean activity coefficients can be directly measured experimentally, but single-ion activity coefficients cannot. Two different assumptions have been made to extract single-ion γ's from mean γ's (MacInnes, 1961, p. 242). The "Guggenheim assumption" is that the cation, anion, and mean activity coefficients for a univalent-univalent salt M^+Cl^- are equal: $\gamma_M=\gamma_{Cl}=\gamma_{MCl}$. The "MacInnes assumption" is that γ_{Cl} is independent of which monovalent cation Cl^- is associated with, and that in a KCl solution $\gamma_{Cl}\doteq\gamma_K\doteq\gamma_{KCl}$ since K^+ and Cl^- have similar electronic structures. The difference between these two assumptions appears most clearly in the case of the biionic junction system $Ag/AgCl:M^+Cl^-$ (150 mM):M^+Cl^- (150 mM) in agar bridge N^+Cl^- (150 mM):Ag/AgCl, in which the two Ag/AgCl electrode potentials would be identical and would cancel by the MacInnes assumption but would yield a p.d. of up to 1 mV by the Guggenheim assumption.

Column 3 of Table 2 gives the circuit p.d.'s measured with Ag/AgCl electrodes before any corrections have been applied. These p.d.'s were next corrected by subtracting the small change in standard state potential E caused by mannitol in the case of dilution junctions and described in the next section and in Fig. 6 (biionic junctions contained no mannitol and thus

Table 2. *Comparison of theoretical and experimental salt bridge junction potentials at 23 °C* [a]

Solutions (mM)		Measured p.d. without corrections	Corrected p.d.		Theoretical p.d. [Eqs. (1) or (2)]
1	2		Mac-Innes	Guggenheim	
LiCl 75	LiCl 150	10.5	5.5	6.0	6.0
NaCl 75	NaCl 150	12.5	3.5	3.7	3.7
KCl 75	KCl 150	15.7	0.4	0.4	0.3
RbCl 75	RbCl 150	16.1	−0.1	−0.2	−0.1
CsCl 75	CsCl 150	15.7	−0.2	−0.5	−0.1
RbCl 150	NaCl 150	4.8	4.8	5.5	5.6
CsCl 150	NaCl 150	4.6	4.6	5.7	5.5
KCl 150	NaCl 150	5.2	5.2	5.7	4.8
LiCl 150	NaCl 150	−2.3	−2.3	−2.9	−2.9
CsCl 120 LiCl 30	CsCl 150	−1.2	−1.2	−1.5	−1.4
CsCl 90 LiCl 60	CsCl 150	−2.3	−2.3	−3.0	−2.9
CsCl 75 LiCl 75	CsCl 150	−3.3	−3.3	−4.2	−3.7
CsCl 60 LiCl 90	CsCl 150	−4.2	−4.2	−5.2	−4.5
CsCl 30 LiCl 120	CsCl 150	−5.4	−5.4	−6.8	−6.3
LiCl 150	CsCl 150	−6.9	−6.9	−8.6	−8.4
NaCl 150	NaCl 75 KCl 75	—	—	—	−2.5
KCl 150	NaCl 75 KCl 75	—	—	—	+2.3
RbCl 150	RbCl 75 CsCl 75	—	—	—	0.0
RbCl 120 CsCl 30	RbCl 75 CsCl 75	—	—	—	0.0
RbCl 90 CsCl 60	RbCl 75 CsCl 75	—	—	—	0.0
RbCl 60 CsCl 90	RbCl 75 CsCl 75	—	—	—	0.0
RbCl 30 CsCl 120	RbCl 75 CsCl 75	—	—	—	0.0
CsCl 150	RbCl 75 CsCl 75	—	—	—	−0.1

[a] Columns 1 and 2 give the principal components of the experimental solutions used (each solution contained in addition 0.25 mM $CaCl_2$ and 2 mM HEPES). Columns 3, 4 and 5 give, respectively, the uncorrected experimental value of the junction potential measured directly with Ag/AgCl electrodes, the value after correction by the MacInnes assumption, and the value after correction by the Guggenheim assumption (*see* p. 104 of the text). Column 6 gives the theoretical value of the junction potential calculated from Eqs. (1) or (2). All p.d.'s are in mV and are stated as the potential of solution 2 with respect to solution 1. Experimental errors in all measured p.d.'s are ± 0.1 or ± 0.2 mV.

did not require this correction). Finally, the p.d.'s were corrected for the Cl^- activity difference at the electrodes, using mean $\gamma_\pm$'s from Robinson and Stokes (1965) and either the Guggenheim or the MacInnes assumption to extract γ_{Cl} from $\gamma_\pm$. The resulting junction potentials are given in column 4 of Table 2 for the MacInnes assumption, and in column 5 for the Guggenheim assumption.

It will be seen from Table 2 that all three methods yield values which agree usually within 1 mV for a given junction. The largest discrepancy is 1.7 mV for the LiCl:CsCl junction. The theoretical values agree somewhat better with measured values using the Guggenheim assumption than with measured values using the MacInnes assumption. In practice, we used the theoretical values to correct the experimental p.d.'s reported in the following papers.

In order to test the reliability of measurements with salt bridges under actual experimental conditions, we carried out three experiments in which several kinds of diffusion potentials were recorded from the same gallbladder, alternately using as recording electrodes either Ag/AgCl electrodes dipping directly into the bathing solutions or salt bridges connected to calomel electrodes. P.d.'s obtained with salt bridges were corrected by

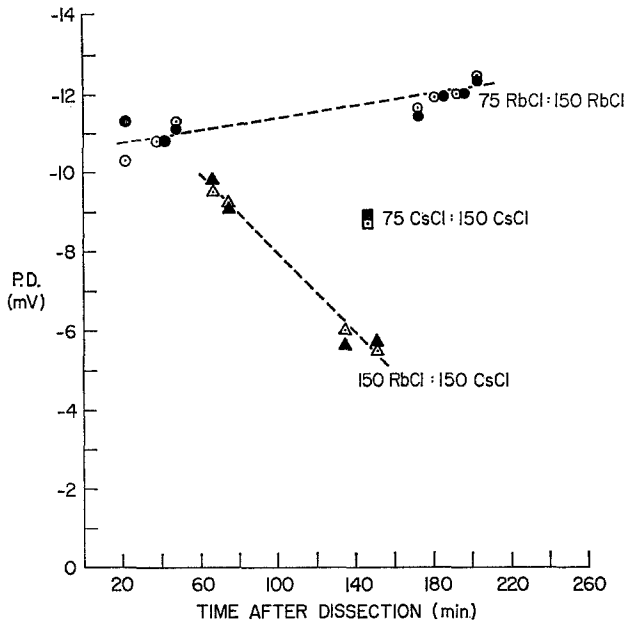

Fig. 5. A comparison of diffusion potentials as measured with Ag/AgCl electrodes (solid symbols) and with 150 mM CsCl or RbCl agar bridges (open symbols) in the same gallbladder. Agar bridge measurements were corrected for the junction potentials listed in the last column of Table 2, whereas Ag/AgCl measurements were corrected for electrode potentials using the Guggenheim assumption and correcting for the effect of mannitol on E_0 (p. 107). Four kinds of p.d.'s were measured: 2:1 RbCl dilution potentials (● or ⊙) 2:1 CsCl dilution potentials (■ or □), biionic potentials with 150 mM RbCl as the serosal solution and 150 mM CsCl as the mucosal solution (▲ or △ at 70 min), and biionic potentials with 150 mM CsCl as the serosal solution and 150 mM RbCl as the mucosal solution (▲ or △ at 140 min, plotted with signs reversed from positive to negative) Note that both recording arrangements yield essentially the same value of the p.d in each case

subtracting the calculated Planck junction potentials, whereas p.d.'s obtained with Ag/AgCl electrodes were corrected by subtracting the difference in electrode potentials calculated from the Guggenheim assumption. As illustrated in Fig. 5, results from the two recording arrangements agree closely.

These considerations of junction potentials may be summarized as follows. Although use of flowing saturated KCl bridges is sufficiently accurate for measuring a large p.d., the error they introduce becomes significant if one requires accurate determination of small p.d.'s, and static saturated KCl bridges would be worse. These difficulties may be minimized by choosing bridges of appropriate composition to give stable, profile-independent junction potentials with the experimental solutions used. The values of these junction potentials may be calculated from Eq. (1) or Eq. (2), and comparison with two different methods of calculation suggests that the uncertainty in these values is generally less than 1 mV.

Effects of Nonelectrolytes on Electrode Standard Potentials and Ion Activities

Many of the solutions used in our experiments contained nonelectrolyte concentrations of 140 mM and in a few cases up to 700 mM. Thus, it was important to know whether these nonelectrolyte concentrations affected electrode standard potentials or ion activities.

A test of the effect on the standard potential of an Ag/AgCl electrode was carried out by means of the circuit Ag/AgCl:0.15 M RbCl + nonelectrolyte:0.15 M RbCl in agar bridge:saturated KCl:calomel electrode. A solution of 0.15 M RbCl was divided into two aliquots, one of which was used as the control solution free of nonelectrolyte, while mannitol or sucrose was added incrementally to the other. The two solutions were brought to the same temperature again, the temperature of the latter solution having been changed by up to 1 °C by the heat of mixing. A backing-off voltage was used to set the potential of the circuit in the absence of added nonelectrolyte to zero, and the p.d. was then measured as a function of the added nonelectrolyte concentration. As illustrated in Fig. 6, the p.d. shifts in the positive direction by about 0.8 mV per 1 M mannitol, and the effect of sucrose is larger (just as expected from the fact that sucrose is larger than mannitol, hence the change in dielectric constant which sucrose causes is larger than that caused by mannitol).

The effect on the standard potential of a K^+-selective glass electrode is larger than on that of an Ag/AgCl electrode, as illustrated in Fig. 7. The circuit used was K^+ electrode:KCl (C_1) + sucrose (C_2):saturated KCl:

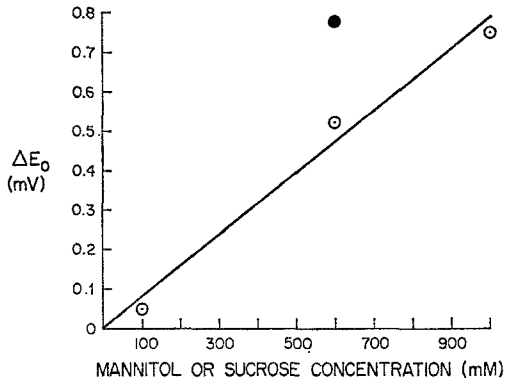

Fig. 6. The change in standard potential, ΔE_0, of an Ag/AgCl electrode in 150 mM RbCl as a function of sucrose or mannitol concentration. The potential of this electrode was measured against that of a calomel electrode connected by saturated KCl and a 150 mM RbCl agar bridge to the solution into which the Ag/AgCl electrode dipped. The ordinate gives the p.d. at the indicated concentration of sucrose (•) or mannitol (⊙) minus the p.d. in the absence of nonelectrolyte. Note that the nonelectrolytes shift the p.d. in the positive direction, and that the effect is larger for sucrose than for mannitol

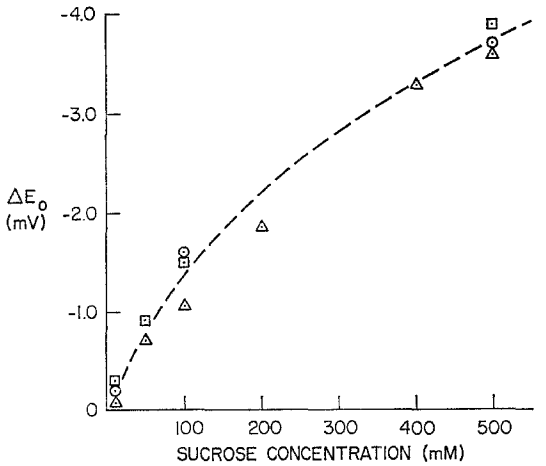

Fig. 7. The change in standard potential of Beckman cation-sensitive electrode no. 39137 in KCl solutions as a function of sucrose concentration. The potential of this electrode was measured against the potential of a calomel electrode connected by a flowing bridge of saturated KCl to the solution into which the cation-sensitive electrode dipped. The solution contained KCl at 1 (⊙), 10 (△), or 100 (⊡) mM plus various concentrations of sucrose and was saturated with $Ca(OH)_2$ to keep the $[H^+]$ low. The ordinate gives the p.d. at the indicated sucrose concentration minus the p.d. in the absence of sucrose but with the same (KCl). Note that sucrose makes the p.d. more negative but that the effect is relatively independent of the KCl concentration, so that it must be the standard potential E_0 rather than an activity coefficient that is changing

calomel electrode. The effect of sucrose added in increments was measured as described in the preceding paragraph, except that solutions were saturated with $Ca(OH)_2$ to buffer them at a high pH and thus to suppress the response of the electrode to H^+. As seen in Fig. 7, 0.5 M sucrose shifts the circuit potential in the negative direction by 3.6 mV, but this shift is independent of KCl concentration. Therefore, the effect must be on a standard potential rather than on an activity coefficient. Similar effects of the nonelectrolyte methanol on the standard potential of K^+-selective glass electrodes were demonstrated previously (Eisenman, 1965).

These effects are presumably mainly on electrode standard potentials rather than on ion standard state potentials, since the changes in activity of both KCl (Robinson & Stokes, 1962) and NaCl (Kelly, Robinson & Stokes, 1961) caused by mannitol are much too small and in the wrong direction to produce the observed p.d. change. Because mannitol has less effect than sucrose on electrode standard potentials, mannitol was the nonelectrolyte used to adjust the osmolality of experimental solutions. The results of Fig. 6 were used to correct E_0 for the effect of mannitol in measuring junction potentials with Ag/AgCl electrodes, as described on p. 104.

One Membrane or Two Membranes?

The anatomy of the gallbladder (*see* Fig. 1) might at first lead one to expect that it would constitute a series two-membrane system, in which permeating ions would have to traverse in turn the membranes at the serosal and mucosal faces of the cells, separated by the intracellular fluid. The transepithelially measured p.d. would then be the sum of two p.d.'s in series. However, the gallbladder might behave as a single-membrane system if the conductance of the tight junctions[1] between cells were much higher than the conductance of the transcellular pathway, or if one of the two cell membranes had a much higher resistance than the other. The same question arises in interpreting the transepithelially measured properties of other epithelia (Ussing & Windhager, 1964; Windhager, Boulpaep & Giebisch, 1967). Since the behavior of the gallbladder in all our experiments appeared consistent with that expected for a single membrane, two kinds of experiments were designed to test specifically whether it was behaving as a one-membrane or a two-membrane system.

1 The expression "conductance of the tight junctions" as used here refers to their longitudinal conductance measured between the external bathing solutions, not to their lateral conductance measured between adjacent cells and responsible for cell-to-cell coupling.

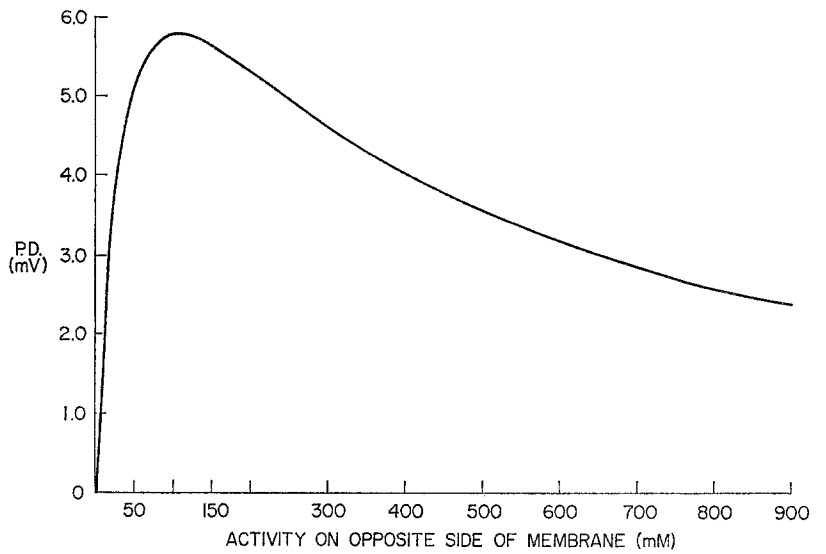

Fig. 8. The predicted change in p.d. (ordinate) across a single membrane when the bathing solution on one side is changed from CsCl at an activity of 150 mM to CsCl at an activity of 75 mM, as a function of the CsCl activity on the opposite side of the membrane (abscissa). This situation is exemplified by measuring 150:75 mM CsCl dilution potentials resulting from mucosal solution changes in rabbit gallbladder, as a function of the serosal solution composition. The predictions were made from the Goldman-Hodgkin-Katz equation (Barry, Diamond & Wright, *in preparation*), taking $P_{Cl}/P_{Cs}=\alpha$ as 0.5. Thus, the ordinate is

$$\Delta E=\frac{RT}{F}\left(\ln\frac{75+\alpha a}{a+\alpha 75}-\ln\frac{150+\alpha a}{a+\alpha 150}\right)=\frac{RT}{F}\ln\frac{(75+\alpha a)(a+150\alpha)}{(a+\alpha 75)(150+\alpha a)}$$

where RT/F was taken as 25.5 mV at 23 °C

The principle underlying both types of experiments is that the *change* in p.d. across a single membrane caused by a given change in salt composition in one bathing solution depends upon the salt composition of the opposite bathing solution, unless the membrane is perfectly permselective. Fig. 8 illustrates this effect for a single-salt dilution potential of a univalent-univalent salt, and gives the calculated *change* in p.d. when the salt concentration on one side of a membrane is changed from 150 to 75 mM, as a function of the salt concentration on the second side. The p.d. change is maximal when the salt concentration on the second side is 106 mM (mean of 150 and 75), and decreases for higher or lower values. It may be shown that the dependence of the percentage change in p.d. on the composition of the second side becomes more marked as the ratio of anion to cation permeability approaches 1.0.

The two experiments based on this principle were as follows:

1. Effect of Serosal Concentrations on P.D. Changes Resulting from Mucosal Concentration Changes

If transepithelially measured properties of the gallbladder were dominated by a single membrane, then the change in p.d. resulting from a change in the mucosal solution composition would depend upon the serosal solution composition, as illustrated in Fig. 8. If, on the other hand, the measured p.d. were the sum of two p.d.'s in series, then the change in p.d. resulting from a change in mucosal composition would be initially independent of the serosal composition. The reason is that the mucosal cell membrane, across which the change in p.d. would arise, would have the relatively constant intracellular fluid rather than the serosal solution on the opposite side, and the gradient across the serosal membrane (intracellular fluid vs. serosal solution) would be initially independent of a change in mucosal composition. The intracellular fluid may, of course, change in composition when the external solutions are changed, but the intracellular changes must lag behind the extracellular ones so that the intracellular fluid will be initially constant.

The actual experiment consistent of measuring the p.d. change for a change in mucosal solution from 150 to 75 mM CsCl, first when the serosal solution was 150 mM CsCl, then when the serosal solution was nominally 18.75 mM CsCl (all solutions being kept isosmotic by replacing CsCl with mannitol). CsCl was chosen both in order to minimize junction potentials and to maximize the possible effect, since the gallbladder's permeability to Cs^+ is closer to that of Cl^- than is that of any other alkali cation (Wright *et al., in preparation*; Barry *et al., in preparation*). The experimental protocol consisted first of setting up the gallbladder with 150 mM CsCl on both sides and measuring the change in p.d. when the mucosal solution was changed back and forth between 150 and 75 mM CsCl. Next, both mucosal and serosal solutions were changed to 18.75 mM CsCl, giving rise to a transient diffusion potential because the unstirred layers at the mucosal and serosal surfaces are of different thicknesses (Diamond, 1966; *see* also p. 96 for discussion), and hence the solutions at the mucosal and serosal surfaces change from 150 to 18.75 mM at different rates. After this transient p.d. had decreased, the mucosal solution was changed to 150 mM CsCl, the change in p.d. was measured as the mucosal solution was changed back and forth several times between 150 and 75 mM, and the mucosal solution was then changed back to 18.75 mM CsCl. Finally, additional measurements were carried out again with the serosal solution back at 150 mM, then at 18.75 mM, and at 150 mM again.

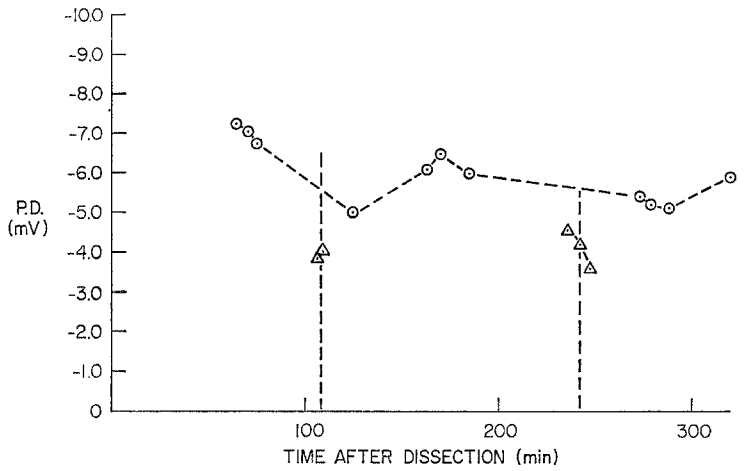

Fig. 9. Effect of serosal concentrations on p.d. changes resulting from mucosal concentration changes. The *change* in p.d. when the mucosal solution was changed from 150 mM CsCl to 75 mM CsCl was repeatedly measured, with either 150 mM CsCl (⊙) or 18.75 mM CsCl (▽) alternately as the serosal solution. At the two times indicated by the vertical lines, the two sets of p.d.'s were compared, using the graphically interpolated values on the curve at 150 mM (⊙). Note that the p.d. change is lower at serosal concentrations of 18.75 mM (▽) than at 150 mM (⊙)

Fig. 9 and comparison of columns 3 and 4 of Table 3 show that the change in p.d. was lower for a serosal concentration of 18.75 mM than of 150 mM, as expected qualitatively for a single-membrane system (Fig. 8), and in contrast to the expectation of a constant change in p.d. derived from a two-membrane system.

In order to compare the results quantitatively with single-membrane predictions, one must take account of the fact that a small p.d. persisted across the gallbladder when the bulk mucosal and serosal solutions were both 18.75 mM CsCl, because the unstirred layer of connective tissue at the serosal face of the epithelium delayed equilibration with the serosal bathing solution. The actual value of the CsCl concentration at the serosal face was estimated by inserting this p.d., plus the value of P_{Cl}/P_{Cs} obtained from a CsCl dilution potential in the same gallbladder, into the Goldman-Hodgkin-Katz equation (discussed in Barry *et al.*, *in preparation*) and solving for the serosal CsCl concentration. Table 3, column 2, gives for four experiments the average value of this estimated concentration, which fell between 26 and 43 mM. From this estimated serosal concentration, the p.d. change expected for a mucosal change of 150 to 75 mM in a single-membrane system was calculated (Table 3, column 5) in the same way used to construct Fig. 8. Comparison of columns 4 and 5 in Table 3 shows that the

Table 3. *Effect of serosal concentrations on p.d. changes resulting from mucosal concentration changes*[a]

Gallbladder no.	Estimated serosal concn. (mM)	Measured p.d. change at 150 mM (mV)	Measured p.d. change at "18.75" mM (mV)	Prediction for single membrane at "18.75" mM (mV)
33	37	11.4	8.9	8.8
35	26	5.6	4.1	4.1
35	29	5.6	4.0	4.3
37	43	6.4	5.0	6.0

[a] The experimental protocol is that described on p. 111 of the text and illustrated in Fig. 9. Briefly, the change in p.d. when the mucosal solution was changed from 150 to 75 mM CsCl was measured several times, first when the serosal solution was 150 mM CsCl, then when the serosal solution was 18.75 mM CsCl, and finally when it was 150 mM CsCl again. Experiment 33 used RbCl rather than CsCl as the mucosal salt but was otherwise identical. Column 4 gives the average measured change in p.d. at a nominal serosal concentration of 18.75 mM, and column 3 gives the average measured change at a serosal concentration of 150 mM, interpolated to the time of the measurement at 18.75 mM. Owing to equilibration delays in the serosal unstirred layer, the actual concentration at the serosal face of the epithelium did not drop all the way to 18.75 mM. Hence, from the measured asymmetry potential when both bathing solutions were nominally 18.75 mM, the actual serosal concentration in this situation was calculated by means of the Goldman-Hodgkin-Katz equation and is given in column 2. If the gallbladder were a single-membrane system, the p.d. change would be lower at "18.75" mM than at 150 mM. From the estimated serosal concentration of column 2, the predicted p.d. change for a single-membrane system was calculated in the same way used to construct Fig. 8 and is listed in column 5. If the gallbladder were a series two-membrane system, the p.d. change would be independent of the serosal concentration, so that the measured p.d. change at a nominal serosal concentration of 18.75 mM (column 4) would be the same as the measured p.d. change at 150 mM given in column 3. Note that the measured p.d. changes of column 4 are in fact consistently lower than the measured values of column 3 (*compare* Fig. 9 for reproducibility and reversibility of the effect) but agree well with the predicted values for a single membrane given in column 5.

experimental p.d. changes are generally in good agreement with this calculation based on a single-membrane system. Hence both the quantitative and the qualitative outcomes of this experiment favor the single-membrane interpretation over the two-membrane interpretation.

2. Modification of Intracellular Concentrations

In the second type of experiment, the intracellular ion concentrations were increased by symmetrically increasing the osmotic pressure of the mucosal and serosal solutions by addition of 200 or 400 mM mannitol. The dilution potential resulting from a concentration gradient of 50 mM CsCl vs.

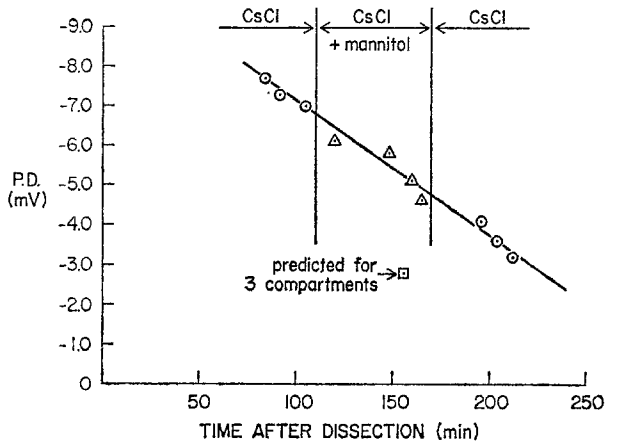

Fig. 10. Effect of intracellular ion concentrations on CsCl dilution potentials. The dilution potential resulting from a CsCl concentration gradient (50 mM CsCl as the serosal solution, 25 mM CsCl as the mucosal solution) was measured repeatedly: first (⊙) in the absence of added mannitol (beyond that required to replace 25 mM CsCl in the mucosal solution osmotically), then with 400 mM mannitol added to both bathing solutions (△), and finally (⊙) in the absence of added mannitol again. The point marked ⊡ is the predicted p.d. for a two-membrane system, calculated as in column 3 of Table 4. Note that added mannitol has no effect on the p.d., contrary to the prediction based on a two-membrane system

25 mM CsCl was then measured as a function of the added mannitol concentration. Estimates of intracellular concentrations (Diamond, 1962*a*) suggest that most of the intracellular osmolality is accounted for by diffusible ions. Hence shrinkage of the cells by addition of 200 or 400 mM mannitol to the bathing solutions should be accompanied by osmotically equivalent increases in intracellular ion concentrations, since mannitol is effectively impermeant (Wright & Diamond, 1969). If transepithelially measured p.d.'s are the sum of the p.d.'s across the two cell membranes in series, then mannitol, by increasing the intracellular ion concentrations, would reduce the 2:1 dilution potential, as illustrated in Fig. 8. However, if the properties of the gallbladder were dominated by a single membrane (e.g., because the high-conductance pathway was across the tight junction and bypassed the cells), mannitol would have no effect on the value of the dilution potential.

As illustrated in Fig. 10, it was found experimentally that addition of 200 or 400 mM mannitol had no effect on the CsCl dilution potential, in accordance with the predictions for a single membrane. The results of three experiments are summarized in Table 4, which shows that the average p.d. expected for a two-membrane system was 2.8 mV, the p.d. expected for a single-membrane system was 4.7 mV, and the measured p.d. was 5.0 mV.

Table 4. *Effect of intracellular ion concentrations on transepithelial dilution potentials*[a]

Gallbladder no.	Mannitol (mM)	Predicted p.d., two membranes (mV)	p.d., no mannitol (mV)	p.d., in mannitol (mV)
45	200	3.3	4.7	5.8
45	400	2.1	3.9	4.5
47	400	2.8	5.2	5.2
67	400	3.0	4.8	4.6
	average:	2.8	4.7	5.0

[a] As discussed in the text and illustrated in Fig. 10, 50 mM:25 mM CsCl dilution potentials were measured, first in the absence of added mannitol, then when the osmotic pressure of both bathing solutions had been symmetrically increased by addition of 200 or 400 mM mannitol, and finally in the absence of added mannitol again. Column 2 gives the added mannitol concentration, column 5 the measured p.d. in the presence of mannitol, and column 4 the average measured p.d. in the absence of mannitol, interpolated to the time of the measurement in the presence of mannitol. In a single-membrane system, mannitol should have no effect on the p.d., i.e., columns 4 and 5 would be identical. In a series two-membrane system, however, the added mannitol would decrease the p.d. by increasing intracellular ion concentrations (*cf.* Fig. 8). To predict the magnitude of this effect, it was assumed that the intracellular fluid could be approximated as a CsCl solution isosmotic with the external bathing solutions, i.e., as 50 mM CsCl in the absence of mannitol, $50+200/(0.9)(2)=161$ mM in the presence of 200 mM mannitol, and $50+400/(0.9)(2)=272$ mM in the presence of 400 mM mannitol (the factor 0.9 is the CsCl osmotic coefficient, and the factor 2 appears because CsCl consists of two osmotically active particles). This approximation neglects the osmotic contribution of fixed intracellular anions, since these are likely to be polyvalent and since gallbladder epithelial cells appear to contain high [Cl^-] (Diamond, 1962*a*). From this estimated intracellular concentration and the experimental value of P_{Cs}/P_{Cl} obtained from dilution potentials, the predicted p.d.'s for a two-membrane system listed in column 3 were calculated in the same way used to construct Fig. 8. Note that the measured p.d.'s of columns 4 and 5 agree well with each other and are consistently higher than the predicted two-membrane values in column 3.

Similarly, addition of 200 or 400 mM sucrose was shown previously not to change NaCl dilution potentials (Diamond & Harrison, 1966, p. 52).

Thus, both of these two types of experiments suggest that the properties of transepithelially measured p.d.'s differ distinctly from those expected for a system of two membranes in series, and cannot be distinguished from those of a single membrane. The results of these two experiments are also consistent with the observation that reversing a salt concentration gradient across the gallbladder yields a diffusion potential of opposite sign but the same magnitude (e.g., Barry *et al.*, *in preparation*, Fig. 3), since this observation will in general not be valid for a series system of two membranes separated by a reservoir (Sandblom & Eisenman, 1967) unless the membranes

happen to have the same relative permeability coefficients. Thus, evidently one of the two cell membranes dominates the transepithelial p.d.'s in the gallbladder, or else the high-conductance pathway is across the tight junction (*see* footnote 1). Other types of experiments in two other epithelia, frog skin (Ussing & Windhager, 1964) and renal proximal tubule (Windhager *et al.*, 1967), have been interpreted to mean that the tight junctions provide the high-conductance pathway. Electron micrographs of epithelial tight junctions (Tormey & Diamond, 1967; Brightman & Reese, 1969) show that they are formed by an apparent fusion of the inner leaflets of the plasma membranes of adjacent cells. The resolution presently obtainable is inadequate to decide whether the epithelial tight junction presents a barrier essentially similar to that faced by substances crossing the membranes of most single cells, or whether it is a basically different structure confined to epithelia. In brief, we cannot be sure at present where the cation permeation mechanism studied in this series of papers resides, but evidence to be present in the following papers favors the tight junctions.

Effects of Unstirred Layers

Immediately adjacent to any membrane separating two well-stirred bathing solutions are unstirred boundary layers. In rabbit gallbladder epithelium, it may be estimated (Diamond, 1966) from half times of diffusion potentials and so-called "streaming potentials" that there is an effectively unstirred layer about 100-μ thick at the interface between the epithelium and the mucosal bathing solution, upon which the epithelial cells abut directly. Between the epithelium and the serosal bathing solution, the connective tissue comprises an unstirred layer about 300-μ thick, in which effective diffusion coefficients are reduced by about 23% below their free-solution values (due presumably to a tortuosity factor).

These unstirred layers in the gallbladder explain (Diamond, 1966) the time course of dilution potentials and streaming potentials resulting from gradients of impermeant solutes, illustrated in Fig. 11a. The salts used in the present papers are permeant to varying degrees, yielding dilution potentials and biionic potentials with a time course illustrated in Fig. 11b (*cf.* also Diamond & Harrison, 1966, Fig. 7). The more permeant the salt used, the more marked is the maximum in the p.d. and the overshoot when the original bathing solution is restored. The reason for this time course is as follows. When the salt concentration in the mucosal solution is suddenly lowered below the level in the serosal solution, the p.d. builds up gradually (Fig. 11a & b) because of the time required for the higher salt concentration

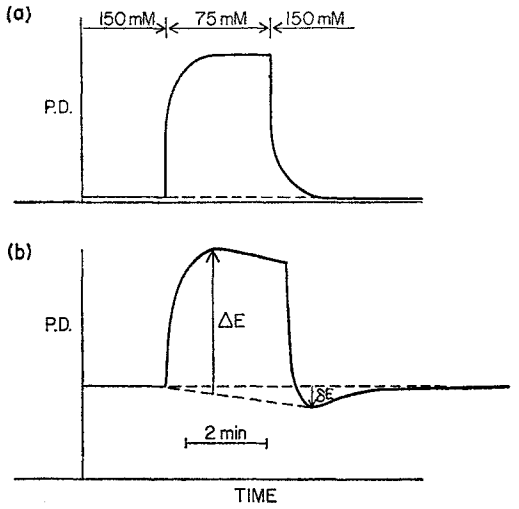

Fig. 11. Typical time courses of diffusion potentials in rabbit gallbladder epithelium, when the serosal solution is maintained at 150 mM salt and the mucosal solution is changed from 150 to 75 mM and back to 150 mM. (a) Gradient of impermeant solute. When the mucosal solution is diluted, the p.d. rises exponentially with a half time determined by the thickness of the mucosal unstirred layer, reaches and remains at a stable value, and returns exponentially without overshoot to zero when 150 mM salt is restored to the mucosal solution. (b) Gradient of permeant solute. The time course differs from the case of the impermeant solute in that the p.d. passes through a maximum after which it slowly declines, and in that the p.d. overshoots zero when 150 mM salt is restored to the mucosal solution. The decay after the maximum is due to salt permeation through the epithelium down its concentration gradient from the mucosal to the serosal solution, lowering the salt concentration at the serosal face of the epithelium below 150 mM. The overshoot (labeled "δE" on the figure) is due to the transient persistence of this lowered concentration at the serosal face of the epithelium. The time courses for biionic potentials and for so-called streaming potentials (Wright & Diamond, 1969) are similar. These unstirred-layer effects were approximately corrected for by reading the value of the p.d. as given by the vertical arrow. The overshoot was generally 0.3 to 6% of this p.d.

initially remaining in the unstirred layer at the mucosal surface to become dissipated by diffusion into the well-stirred mucosal solution. As a salt concentration gradient builds up across the epithelium, salt begins to diffuse from the serosal surface of the cells through the epithelium to the mucosal surface, at a rate depending upon the salt's permeability. This continued permeation through the epithelium reduces the effective local transepithelial concentration gradient, by lowering the salt concentration in the serosal boundary layer below the bulk serosal value and raising the concentration in the mucosal layer above the bulk mucosal value. The effect in the thicker serosal layer is quantitatively much more important. Thus, the local trans-

epithelial concentration gradient, and hence the p.d., goes through a maximum (Fig. 11 b). When the original salt concentration is restored in the mucosal bathing solution, the p.d. does not return smoothly to zero but overshoots (Fig. 11 b), because the salt concentration in the serosal boundary layer is initially still below the bulk serosal bathing solution value. As expected, these transient effects are more marked for the more permeant salts, and more marked for cation biionic potentials in general than for dilution potentials, since the effects are proportional to ion permeabilities. Provided that the decay of the p.d. after passing the maximum is slow compared to the buildup rate, the extrapolation procedure illustrated in Fig. 11 b permits an estimate of the p.d. corrected for unstirred-layer effects, and this procedure was used in this series of papers to correct p.d.'s resulting from gradients of the more permeant salts or ions. The magnitude of the correction was generally in the range 0.3 to 6%, so that it is of limited quantitive significance.

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Appendix

Junction Potential Equations

The Henderson and the Planck integrations of the Nernst-Planck flux equations for a liquid junction relate the junction potential to concentrations rather than to activities. The derivations tacitly assume either that activities are equal to concentrations (activity coefficients equal 1.0) or that activity coefficients are concentration-independent and constant through the junctional profile (*cf.* MacInnes, 1961, p. 233, footnote 16). In this appendix, we derive somewhat more general junction potential equations, which make neither of these assumptions, for the dilution junction and the biionic junction.

Dilution Junction. Consider a junction formed by two solutions of the same univalent-univalent salt at different concentrations.

We symbolize activities by a, concentrations by C, activity coefficients by γ, fluxes by J, mobilities by u, the electrical potential by ψ, and the Faraday by F. Subscripts 1 and 3 refer to the cation and to the anion, respectively. Superscripts $'$ and $''$ refer to the two bathing solutions. By

electroneutrality, $C_1(x)=C_3(x)$ at any distance x along the junctional profile.

We assume that $\gamma_1(x)=\gamma_3(x)$ (the Guggenheim assumption), but it is not assumed that γ is concentration-independent or constant through the junction. From the electroneutrality condition and the Guggenheim assumption, it follows that $a_1(x)=a_3(x)$. u_1 and u_3 are assumed independent of concentration. It is not assumed that the junctional profile has reached the steady state; concentrations may still be functions of time.

The Nernst-Planck flux equations ($J_i=-C_i u_i \frac{\partial \mu_i}{\partial x}$, where μ_i is the electrochemical potential of the i^{th} ion) may then be written as:

$$J_1=-u_1 RT \frac{C_1}{a_1}\frac{\partial a_1}{\partial x}-u_1 C_1 F \frac{\partial \psi}{\partial x}, \tag{3}$$

$$J_3=-u_3 RT \frac{C_3}{a_3}\frac{\partial a_3}{\partial x}+u_3 C_3 F \frac{\partial \psi}{\partial x} \tag{4}$$

where ψ, $C_1=C_3$, and $a_1=a_3$ (and J_1 and J_3 in the nonsteady state but not in the steady state) vary with x.

At zero current $J_1=J_3$, and one may equate Eqs. (3) and (4):

$$-u_1 RT \frac{C_1}{a_1}\frac{\partial a_1}{\partial x}-u_1 C_1 F \frac{\partial \psi}{\partial x}=-u_3 RT \frac{C_3}{a_3}\frac{\partial a_3}{\partial x}+u_3 C_3 F \frac{\partial \psi}{\partial x}. \tag{5}$$

Since $C_1=C_3$ and $a_1=a_3=a$, Eq. (5) yields:

$$-u_1 RT \frac{\partial a}{\partial x}-u_1 a F \frac{\partial \psi}{\partial x}=-u_3 RT \frac{\partial a}{\partial x}+u_3 a F \frac{\partial \psi}{\partial x}$$

or

$$\frac{\partial \psi}{\partial x}=\frac{-RT}{F}\frac{(u_1-u_3)}{(u_1+u_3)}\frac{\partial \ln a}{\partial x}. \tag{6}$$

Integrating Eq. (6) from solution ′ to solution ″ yields Eq. (1) given on p. 102 of the text for the junction potential of a dilution junction:

$$E_L \equiv \psi''-\psi'=\frac{-RT}{F}\frac{(u_1-u_3)}{(u_1+u_3)}\ln\frac{a''}{a'}. \tag{1}$$

Eq. (1) has the same form as the Henderson or Planck equations for a dilution junction but contains activities instead of concentrations. Eq. (1),

hence the dilution junction potential, is independent of time and the junction concentration profiles.

Biionic Junction. Consider a junction involving two monovalent cations (subscripts 1 and 2) and a single monovalent anion (subscript 3), for instance, NaCl vs. KCl, or LiCl-CsCl vs. LiCl.

By electroneutrality, $C_1(x)+C_2(x)=C_3(x)$.

We assume that $\gamma_1(x)=\gamma_2(x)=\gamma_3(x)\equiv\gamma(x)$ (an extended Guggenheim assumption), but γ may be concentration-dependent and may vary through the profile.

The Nernst-Planck flux equations take the form:

$$J_1=-u_1 RT\frac{C_1}{a_1}\frac{\partial a_1}{\partial x}-u_1 C_1 F\frac{\partial\psi}{\partial x}, \tag{7}$$

$$J_2=-u_2 RT\frac{C_2}{a_2}\frac{\partial a_2}{\partial x}-u_2 C_2 F\frac{\partial\psi}{\partial x}, \tag{8}$$

$$J_3=-u_3 RT\frac{C_3}{a_3}\frac{\partial a_3}{\partial x}+u_3 C_3 F\frac{\partial\psi}{\partial x}. \tag{9}$$

Multiplying Eqs. (7)–(9) by $\gamma(x)$ yields:

$$\gamma J_1=-u_1 RT\frac{\partial a_1}{\partial x}-u_1 a_1 F\frac{\partial\psi}{\partial x}, \tag{10}$$

$$\gamma J_2=-u_2 RT\frac{\partial a_2}{\partial x}-u_2 a_2 F\frac{\partial\psi}{\partial x}, \tag{11}$$

$$\gamma J_3=-u_3 RT\frac{\partial a_3}{\partial x}+u_3 a_3 F\frac{\partial\psi}{\partial x}. \tag{12}$$

At zero current,

$$J_1+J_2=J_3 \quad \text{or} \quad \gamma J_1+\gamma J_2=\gamma J_3. \tag{13}$$

The problem is now formally identical to the initial equations of the Planck or Henderson derivations, except that activities now appear in place of concentrations.

In analogy to the Henderson mixture assumption for concentrations (MacInnes, 1961, p. 231), we now assume that the activities at any point in the junctional profile may be expressed as a mixture of the activities in the bulk solutions $'$ and $''$:

$$\begin{aligned} a_1(x)&=a_1'+(a_1''-a_1')\,y \\ a_2(x)&=a_2'+(a_2''-a_2')\,y \\ a_3(x)&=a_3'+(a_3''-a_3')\,y \end{aligned} \tag{14}$$

where y is the mixing fraction of the bulk solutions and varies from 0 to 1 as one proceeds from solution ′ to solution ″.

Adding Eqs. (10) and (11) and subtracting Eq. (12), making use of Eq. (13), yields, on rearranging:

$$\frac{\partial \psi}{\partial x} = \frac{-RT}{F}\left(\frac{1}{u_1 a_1 + u_2 a_2 + u_3 a_3}\right)\frac{\partial}{\partial x}(u_1 a_1 + u_2 a_2 - u_3 a_3). \tag{15}$$

By substitution of Eq. (14), Eq. (15) may be integrated from solution ′ to solution ″ in terms of the mixing fraction y instead of in terms of x:

$$E_L = \psi'' - \psi' =$$

$$\frac{-RT}{F}\int_0^1 \frac{[u_1(a_1''-a_1')+u_2(a_2''-a_2')-u_3(a_3''-a_3')]\,dy}{u_1 a_1' + u_2 a_2' + u_3 a_3' + y[u_1(a_1''-a_1')+u_2(a_2''-a_2')+u_3(a_3''-a_3')]}$$

or

$$E_L = \frac{-RT}{F}\left(\frac{u_1(a_1''-a_1')+u_2(a_2''-a_2')-u_3(a_3''-a_3')}{u_1(a_1''-a_1')+u_2(a_2''-a_2')+u_3(a_3''-a_3')}\right)\ln\frac{u_1 a_1'' + u_2 a_2'' + u_3 a_3''}{u_1 a_1' + u_2 a_2' + u_3 a_3'}.$$

When $a_1'' = 0$, $a_2' = 0$, and hence $a_1' = a_3'$ and $a_2'' = a_3''$, Eq. (16) reduces to Eq. (2) of the text for the junction potential of a biionic junction:

$$E_L = \frac{-RT}{F}\left[\frac{a_2''(u_2-u_3)-a_1'(u_1-u_3)}{a_2''(u_2+u_3)-a_1'(u_1+u_3)}\right]\ln\frac{a_2''(u_2+u_3)}{a_1'(u_1+u_3)}. \tag{2}$$

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